EXHIBIT A

High-Amylose Corn Starch Fractions

By Edna M. Montgomery, K. R. Sexson and F. R. Senti, Peoria, Illinois (USA)

Dedicated to Professor Dr. M. Samec, Ljubljana, on the occasion of his 80th birthday

Introduction

New varieties of corn starch containing increased levels of the amylose or linear component, as indicated by iodine binding of the starch, have been obtained recently as a result of genetic developments in corn (4, 5, 7, 14, 16). Establishment of the high amylose content by isolation of the linear component was reported for one corn starch (15). Levels of amylose in the new starches far exceed the 27 % found in ordinary or dent corn starch. As a result, new basic information is needed to evaluate the effect of the altered granule composition on the character of the starch and of its amylose and amylopectin components. In order to compare high-amylose corn starch and its fractions to dent corn starch and its fractions a series of eight inbred and two hybrid corn starches with six levels of amylose content have been fractionated into their respective amylose and amylopectin components. The high-amylose starches evaluated ranged in „apparent amylose" content, as shown by iodine affinity, from 50 to 71 % and were isolated by wet-milling procedure (1) after either steeping at 37° C in distilled water or at 50—60° C in distilled water containing sulfur dioxide.

Evaluation of such a series of corn starches should furnish some evidence of trends in properties as the amylose content of the starch granule increases. However, a consistent relationship cannot be predicted because this series is not extensive enough to represent all genotype and interaction effects. Various gene combinations can give the same amylose content but may introduce differences in starch structure just as differences occur in the corn kernel characteristics (7, 14a, 14b).

Materials and Methods

Hybrid high-amylose corn samples were from commercial generation corn of amylomaize types developed and supplied by the Bear Hybrid Corn Co., Inc.[1]) The inbred corns were supplied from breeding studies by M. S. Zuber and associates.

High-amylose corn starches were prepared by wet-milling procedure after either steeping in water at 37° C for 24 hours or steeping in water containing SO_2 at 50° C to 60° C for 48 hours. The starches were dried in a mechanical convection oven at 40° C or 49° C. The dried starch contained from 7 to 10 % moisture and from 0.5 to 1.5 % protein. A total of 10 starches were investigated. The preparations, isolated in lots of 80 g to a few kg in size, were reasonably free of extraneous material and, with a few exceptions, were not more than a few months old when used.

Phosphate buffer contained 50 ml 0.2 M KH_2PO_4 and 17.8 ml 0.2 M KOH diluted to 200 ml; for use 1 ml of buffer was diluted to 100 ml. Ethanol was U.S.P. ethyl alcohol, 99 % anhydrous. n-Butanol was of reagent grade, redistilled. Glycerol was of reagent grade. Pentasol was Sharples Brand Pentasol 27, repurified by shaking with Darco G 60 decolorizing carbon then filtering through analytical grade Celite.

Potentiometric iodine titrations were made according to Bates, French and Rundle as modified by Wilson, Schoch and Hudson (3). For calculation of purity of corn amylose, „pure" amylose is assumed to absorb 200 mg I_2/g. Intrinsic viscosity was measured in 1 N potassium hydroxide according to Lansky et al. (8), but measurements were made at 25 ± 0.06° in place of 35.0° C as reported by these authors. Spectrophotometric analysis of iodine binding of amylose and amylopectin was made according to the method of Melvin and Glass (10); 100 % amylose corresponded to a sample having an iodine affinity of 200 mg I_2/g. β-Amylase was crystalline sweet potato amylase prepared according to Balls et al. (2a) and was free of α-amylase as shown by the method of Olson et al. (2b). Reducing power of maltose was determined according to the method of Somogyi (13) by spectrophotometric measurement of optical density at 7700 Å. Concentration of carbohydrates in aqueous solution was determined by polarimetric measurement or by anthrone method (12).

Solutions and reaction mixtures were deaerated by passing a lively stream of nitrogen through a fritted-glass disk into the mix and allowing the gas to exit under water. Solutions and mixtures were deaerated for 30 minutes prior to use and during all subsequent operations. Nitrogen free of oxygen was obtained by purification of tank gas of 99.5 % purity according to the method of Meites and Meites (9).

Molecular weight by light scattering of amylose was measured in aqueous solution containing 1 % ethanol, p_H controlled at 4.3 to 4.5 with sodium acetate-acetic acid buffer. Crystalline amylose was first dispersed in phosphate-buffered boiling water under nitrogen in presence of n-butanol; n-butanol was removed with steam completely (as shown by alkoxyl determination). Amylopectin solutions were prepared by dissolving this fraction in deaerated 0.5 N sodium hydroxide at 25° C and neutralizing the solution to p_H 6.5.

Separation of Amylose from Amylopectin of High-Amylose Corn Starch After Granule Pretreatment in Aqueous n-Butanol-Glycerol Mixture at 98° C

The separation of high-amylose corn starch into fractions of amylose 1, amylose 2, and uncontaminated amylopectin required 12 steps. The procedure can be conveniently carried to the first precipitation of amylose 1 as the n-butanol complex in one working day. For granule pretreatment a 20-g sample (dry weight) of 60-mesh sieved starch was suspended at 5 % concentration in a mixture of 30 % distilled water, 35 % n-butanol, and 35 % glycerol by weight contained in a round-bottomed Pyrex distilling flask fitted with

[1]) The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

a thermometer, gas-tight mechanical stirrer, condenser, gas inlet tube extending into the reagent mix, and gas outlet through the condenser. The mixture was stirred and deaerated while the temperature was raised to 98°C during 20 to 30 minutes. Heat was controlled by a bath of water containing 5 % of common salt. The temperature of the mixture of solvent and starch was maintained at about 98°C for 3¼ hours; the granules lost all birefringence and swelled to from two to four times their original size.

The high-boiling solvents were removed from the pretreated starch by washing it in 98 % ethanol at about 25°C. Prior to washing, the hot starch-solvent mixture was cooled in the flask in an ice-water bath in a few minutes to about 30°C. The cool mixture was then stirred in 2½ liters of ethanol for 10 minutes; starch was separated by filtration under a rubber dam and removed from the filter. Three additional washes in ethanol, each with 10 minutes of stirring, were required to remove all of the high-boiling solvents as shown by analyses of the starch. Longer contact of the pretreated starch with ethanol, e.g. several hours, was avoided.

After the last filtration the washed starch was weighed to estimate the ethanol content and slurried in sufficient additional ethanol to make a total solvent weight of 150 g. The starch was then further swollen by adding the slurry to 250 ml of 3 N carbonate-free deaerated sodium hydroxide at 10°C. The mixture was agitated slowly with a magnetic stirrer and kept at 10°C for ½ hour in a 5-liter, 3-necked Pyrex distilling flask. Dispersion of the starch at 10°C in 0.5 N sodium hydroxide containing 10 to 12 % by weight of ethanol was carried out by drawing 1,200 ml of deaerated water containing phosphate buffer at 10°C into the flask containing the starch swollen in the alkali-alcohol solvent and by shaking the whole at a moderate rate mechanically for 10 to 15 minutes. The tenderized, swollen granules appeared to collapse while the amylose was extracted very rapidly accompanied by heavy foaming from the nitrogen passed through the dispersion.

For initial separation of amylose from the amylopectin, the alkaline dispersion was neutralized at 20°C to 25°C to p_H 6.5 to 6.6 with 5 N hydrochloric acid added slowly, preferably from a burette, and the mixture was sedimentated at 3700 X g for 30 minutes (25° to 35°C).

The presence of ethanol interfered with direct determination of the yield and purity of the amylose in the supernatant by iodine potentiometric titration; instead analyses were made on isolated materials. The amylose-containing supernatant was decanted through a coarse, fritted-glass filter into a Pyrex flask containing an excess of n-butanol, and the mixture was refluxed for 30 minutes. Amylose separated as the butanol complex from the solution as it cooled overnight in the flask to 25°C.

The crystalline complex represented the crude amylose 1 fraction. The purity of the isolated solvent-free amyloses, as shown by iodine affinity, varied from 92 to 95 % of that of „pure" dent corn amylose (200 mg I_2/g). Yields of amylose estimated by comparison of the total iodine affinity of the isolated fraction with the iodine affinity of the amylose estimated to be present (80 % of the total iodine affinity of the parent starch) ranged from 92 to 96 %.

Purification of amylose 1 was made by two or three recrystallizations in about 1 % concentration in aqueous butanol, p_H adjusted to 6.5 with phosphate buffer according to the conditions of the first crystallization. Prior to measurement, amylose was separated from aqueous butanol by centrifugation and washed in several volumes of anhydrous methanol. After three solvent changes, in one of which the amylose stood overnight, the amylose was recovered by filtration under a rubber dam, spread on a watch glass, and most of the solvent removed by storing overnight over $CaCl_2$ in an evacuated desiccator. Finally the amylose, free of lumps, was stored at 25°C in 50 % R.H. to come to constant weight as the last of methanol was replaced by moisture. The amylose usually contained 10 to 11 % moisture and was not hygroscopic. The intrinsic viscosity of the amylose in 1 N KOH was

Table 1. Amylose Fractions from High-Amylose Corn Starches

Breeding	Starch		Yield			Iodine Affinity Mg I_2/g	[η] 1 N KOH	MWLS[2]
	% „Apparent Amylose"	Steep	Fraction	% Starch	% Total Amylose[1]			
Hybrid	50	H_2O	1	36	91	201	1.36	
			2	3	9	165		
Hybrid	57	SO_2	1		93	206	1.33	283,000
			2		7	168		
Inbred	61—65	H_2O	1	45	90	205	1.37	
			2	5	10	170		
Inbred	61—65	SO_2	1		91	200	1.32	
			2		9	172		
Inbred	66	H_2O	1	48	92	202	1.35	
			2	4	8	169		
Inbred	68	SO_2	1		91	200	1.35	
			2		9	170		
Inbred	71	H_2O	1	50	89	206	1.37	334,000
			2	6	11	175		
Hybrid	27	H_2O	1[3]		90	200	1.36	312,000

[1]) Percent of total amylose isolated as the crystalline n-butanol complex, the total being about 80 % of the „apparent amylose" measured by iodine affinity of the parent starch.
[2]) Molecular weight by light scattering.
[3]) Separated according to MONTGOMERY and SENTI (11).

1.33 to 1.37 for yields of 90 to 93 % of amylose and had a purity of 100—103 % (± 2 %) (Table 1).

Other than for the first crystallization, mother liquors from crystallization of the n-butanol-amylose 1 complex separated at 25° C gave negligible precipitates when chilled to 0°—2° C in a bath overnight or on addition of ethanol. Starch remaining in the mother liquors of the first crystallization was precipitated by addition of four to six volumes of ethanol or until no optical rotation was observed in the aqueous alcohol phase. The precipitate was separated by filtration and added to the principal starch residual or gel from the initial sedimentation of the aqueous starch dispersion.

Amylopectin was separated from the total starch residual after solution in alkali. This residual was covered with 1,000 ml of water containing phosphate buffer at 25° C. One hundred milliliters of deaerated 5.0 N carbonate-free sodium hydroxide was added, together with 50 ml of ethanol, and the mixture was moderately shaken mechanically for 30 minutes. The resulting dispersion, free of solid and lumps, was stored at 0° to 2° C for 24 hours, neutralized at 25° C, refluxed for 15 minutes with an excess of Pentasol, and allowed to cool to 25° C. The Pentasol-amylose complex was removed by sedimentation and purified as amylose 2.

In order to avoid retrogradation of amylopectin, the supernatant was tested for the presence of starch precipitable by Pentasol at 0°—2° C but not at 25° C (a) by diluting the supernatant 1 : 5 with water and chilling the solution with excess of precipitant for 4 hours and (b) by chilling the supernatant to 0° to 2° C for 30 minutes. No insoluble complex separated in either test. The supernatant was then diluted with stirring with excess of ethanol; the precipitate was collected by sedimentation and dissolved at once in 400 ml of buffered water by bringing the mixture to a boil for 15 minutes. On cooling for 30 minutes, a small amount of protein and extraneous material that separated was removed by sedimentation. To remove salts, the amylopectin solution was dialyzed at 25° C in cellophane bags against about 10 volumes of water containing 5 % ethanol with 2 to 3 changes of dialyzing solvent.

When precipitated from aqueous solution with ethanol, the dialyzed amylopectins separated in finely divided form and were collected by centrifugation at 2,000 X g. Sedimented amylopectins were methanol washed, dried, and equilibrated according to the procedure for amylose. Amylopectin was obtained in yields of 90 to 95 % of the weight of starch minus weight of amylose isolated; nitrogen content was less than 0.008 %.

Amylose 2 (Table 1), of iodine affinity lower than that of amylose 1, was isolated as described above from the total crude amylopectin fraction as the Pentasol-amylose complex. The Pentasol-amylose complex was then recrystallized twice from aqueous n-butanol cooling each time to 0° C. As shown in Table 1, the solvent-free fractions had iodine affinities of 165 to 175 mg I_2/g. A subfractionation during recrystallization of one such fraction from 71 % amylose starch is included in Table 2 as amylose 2. A first subfraction (a) was obtained by cooling the hot crystallizing mix to 25° C and a second subfraction, by cooling the mother liquors to 0° to 2° C. The second or more soluble fraction was then recrystallized (b). Concentration of the mother liquors yielded a very small noncrystalline fraction (c).

Table 2. n-Butanol Precipitable Fractions of 71 % Amylose Corn Starch

		Yield % Total Amylose	Iodine Affinity Mg I_2/g	[η] 1 N KOH
Amylose 1		89	206	1.37
Amylose 2	(a)	4.3	180	1.34
	(b)	3.8	168	1.31
	(c)	2.6	138	1.37
		—	—	—

β-Amylase-Conversion of High-Amylose Corn Amylopectin

Amylopectin from 71 % amylose starch (57 % isolable amylose) was converted to maltose by crystalline sweet potato β-amylase to the extent of 75 %. A sample of 4.2 g (dry basis) of fraction was dispersed at 25° C in 100 ml of deaerated 0.5 N sodium hydroxide, neutralized to p_H 6.5 with hydrochloric acid, and adjusted with sodium acetate-acetic acid buffer to p_H 4.3. The volume was adjusted to 250 ml, the concentration of carbohydrate by anthrone method was found to be 1.68 %, and the temperature was brought to 30° C by water bath. Conversion of amylopectin to maltose was made by addition of 10 units (KNEEN and SANDSTEDT) of amylase per gram in presence of toluene. Progress of hydrolysis to maltose measured by reducing power method was 50 % after 12 hours and 75 % after 26 hours; no further change occurred during 20 additional hours although more amylase was added.

β-Amylase limit dextrin was recovered from 150 ml of solution. Temperature of the solution was brought to 95° C to 98° C for 3 minutes; the solution was cooled, dialyzed, and then diluted with excess of ethanol. The precipitated dextrin, dehydrated in methanol, freed of solvents, and equilibrated at 25° C and 50 % R. H., weighed 0.669 g (0.605 g.d.b.) or about 95 % of the expected amount. The dextrin gave no inflection during potentiometric titration.

Amylopectin from 68 % amylose water-steeped starch (54 % isolable amylose) was converted to maltose by β-amylase to the extent of 73 %.

Variations on the Procedure for Separation of Amylose from Amylopectin of High-Amylose Corn Starch.

In Table 3 are recorded results from five experiments in which amylose extraction was made without aqueous-organic solvent pretreatment, length of alkali treatment of the starch was varied, and the amount of non-Pentasol-complexing carbohydrate was determined in the supernatants after low and high speed centrifugation of the starch extracts.

In Exp. 1 the aqueous-organic solvent pretreatment of the granular starch was omitted from the established procedure; only 25 % of the amylose present was extracted. In Exp. 2 corn starch of 50 % apparent amylose content was fractionated according to the established procedure; 94 % of the total amylose was recovered from the centrifugal supernatant of the neu-

Table 3. Separation of Amylose from Amylopectin of High-Amylose Corn Starch by Sedimentation of Neutralized (p_H 6.6) Alkaline Starch Dispersions

Exp. No.	Starch: Steep	Starch: % Apparent Amylose	Pretreatment: HO-glycerol-n-butanol, 98 °C	Swelling Period in 3 N NaOH + Ethanol 10 °C	Extraction Period 0.5 N NaOH + Ethanol	Sedimentation of Starch Dispersions p_H 6.6	Yield of Starch in Principitate[1]: Total	Yield of Starch in Principitate[1]: Pentasol Complex	Yield of Starch in Supernatant: Total[2]	Yield of Starch in Supernatant: Non-complexing	Yield of Starch in Supernatant: Complex Forming	Iodine Affinity of Amylose[4] Mg I_2/g	Yield, % Total Amylose Present[3]
1	H_2O, 37 °C	66	No	30 min.	15 min.	30 min., 3,700 X g	65		35	20	14	176	25
2	H_2O, 37 °C	50	Yes	30 min.	15 min.	30 min., 3,700 X g	48	2	48	9	39	190	94
3[5])	H_2O, 37 °C	50	Yes	30 min.	15 min.	3 hrs., 40,000 X g	58	1	39	—	38	195	96
4	H_2O, 37 °C	50	Yes	60 min.	20 min.	30 min., 3,000 X g	None		98	58	39	187	91
5	H_2O, 37 °C	71	Yes	30 min.	15 min.	30 min., 3,700 X g	35	2	63	6	56	190	94
6	H_2O, 37 °C	61—65	Yes	30 min.	15 min.	3 hrs., 40,000 X g	50	1	50	—	48	194	94

[1]) % Starch.
[2]) Determined by polarimetric measurement.
[3]) Amylose from original supernatant by n-Butanol precipitation.
[4]) On basis of iodine affinity.
[5]) Part of Exp. 2.

tralized starch dispersion after sedimentation at 3700 X g for 30 minutes. The supernatant also contained 9 % of noncomplexing starch or amylopectin. In contrast, a fairly sharp separation of amylose from amylopectin was attained in Exp. 3 by sedimenting a part of the dispersion from Exp. 2 at 40,000 X g for 3 hours.

Investigation of the swelling of the pretreated starch at 10 °C in 3 N sodium hydroxide in the presence of ethanol was made in Exp. 4. Observed microscopically the granules swelled six to eight times in size during 30 minutes without granule destruction, but no appreciable swelling occurred during the succeeding 30 minutes. As shown in Exp. 4, the starch which had been kept in the swelling mix for 1 hour apparently was completely solubilized during a brief extraction period.

In Exp. 5 corn starch of the highest iodine affinity (142 mg I_2/g) to be investigated was fractionated satisfactorily according to the general procedure. Exp. 6 showed that at least for corn starch of an intermediate iodine affinity (126 mg I_2/g), as well as for the 50 % amylose starch, separation of amylose from amylopectin was roughly quantitative if sedimentation of the neutral starch dispersion was made at 40,000 X g for 3 hours.

Discussion of Results

The unusual resistance to gelatinization, swelling, and pasting of high-amylose starches appears to be responsible for the difficulties in fractionating high-amylose corn starch as recorded by earlier workers (15) since their fractionation procedure depended on complete dispersion of the starch. The difficulty of dispersion could, in turn, be ascribed to new associative and secondary bonding forces within the granule brought about by structural changes in the high-amylose starches and/or alteration of the component-content.

A fractionation procedure that is not dependent on complete dispersion would seem preferable for high-amylose starch. Such a procedure was developed for dent corn starch by MONTGOMERY and SENTI (11). They found that pretreatment of undegraded dent corn starch in certain hot organic solvents altered the solution properties of the granule sufficiently to permit sharp separation of amylose and amylopectin by an extraction-sedimentation method, although such a fractionation was not possible in the case of untreated starch. Apparently, changes in the secondary bonding in the granule brought about by the pretreatment loosened amylose from the amylopectin so that amylose was extracted, unentangled with amylopectin, in the hot water. Stronger secondary bonding within the amylopectin kept this component strongly aggregated and water-insoluble, readily separated from the amylose in solution by sedimentation. Amylose was extracted in water at 98 °C in three successive operations, reasonably quantitatively, from 37 °C water-steeped dent corn starch which had been subjected to a prescribed treatment in aqueous organic solvents. Amylopectin, although relatively insoluble in water, was readily disaggregated in alkali to a water-soluble state.

Fractionation Procedure for High-Amylose Starch.

Application of the fractionation procedure (11) to high-amylose corn starch revealed that when the apparent amylose content exceeded 50 %, recovery of the amylose fraction was incomplete and became ever lower as the amylose content of the granule increased. As in the case of dent corn starch, pretreatment of high-amylose corn starches in 6:7:7 water-glycerol-n-butanol mixture at 98 °C for 3½ hours brought about complete loss of birefringence, but contrary to dent starch, granule swelling was, in many starches, rather small. Suspension of the pretreated alcohol-washed starch in either water at 98 °C or 0.5 N sodium hydroxide at 10 °C failed to swell many of the starches appreciably or extract a major portion of the amylose.

The pretreatment-extraction-sedimentation method of fractionation, however, was applied with success to high-amylose corn starches after a mixture of alcohol and 3 N sodium hydroxide in place of water was used to swell the starch and alcoholic 0.5 N sodium hydroxide to extract the amylose. These solvents also served to solubilize the proteins in the starches.

Suspension of the solvent-pretreated starches for ½ hour at 10 °C in 3 N sodium hydroxide containing ethanol resulted in a six- to eightfold swelling without

granule destruction. Amylose was readly extracted from the swollen starch in 0.5 N sodium hydroxide at 10°C in the presence of ethanol. Separation of amylopectin from amylose was effected by sedimentation of the neutralized starch dispersion at 3700 X g for 30 minutes. As a result of centrifugation, an amylopectin fraction separated as a very dense gel; whereas the major linear fraction, amylose 1 of high iodine affinity, was found in the supernatant and was separated as the n-butanol complex and readily purified (Table 1). The alkaline treatment had partially destroyed the bonding which held the amylopectin in aggregated state, and a small part of this component was found in the supernatant (Exp. 2 and 5, Table 3). The starch residual from the amylose 1 crystallization was added to the major residual and the total separated into the more soluble amylose 2 (Tables 1 and 2) and amylopectin (Table 4). Analyses showed the starch fractions to be free of protein.

Effect of protein in the parent starch on the separation of amylose and amylopectin is uncertain. In practice the protein remained in the starch residual or crude amylopectin and was separated with great difficulty unless some treatment, such as that with alkali, was used in the fractionation. In turn, good separations of amylose and amylopectin were coincidental with good separation of protein and isolation of amylopectin free of nitrogen.

The importance of the granule pretreatment as a means of loosening the amylose from amylopectin prior to the separation of the components in alkali is exemplified in Exp. 1 recorded in Table 3. The yield of amylose from the untreated starch was only 25% of that present in contrast to yields from 92 to 96% from pretreated starches in Exp. 2, 3, 5, and 6. Confirmation that good dissociation of components was brought about by the pretreatment is found in the relatively sharp separation of amylose and amylopectin in Exp. 3 and 6. Sedimented amylopectin gel was of small volume and contained little amylose. In Exp. 4 as a result of prolonging the starch swelling period, apparently, complete solution of one starch was obtained and centrifugation at 3,000 X g for 30 minutes did not effect a separation. Amylose might be separated by butanol precipitation from solutions prepared as in Exp. 4 and this fractionation procedure deserves further investigation.

Throughout the work, steps were taken to avoid degradation of the starch. These precautions included p_H control, deaeration of solutions, and use of low temperatures, together with a minimum of agitation in alkali. Use of ethanol in the swelling, extracting, neutralizing, and sedimenting operations served to prevent retrogradation. At this time it cannot be proved whether or not it helped to preserve the starch from degradation.

Demonstration of the yield of amylose was important since it was necessary to make an accounting of the iodine binding of the parent starch. Total starch retrieved as amylose and amylopectin during better runs was 95 to 96%. The amylose fraction accounted for about 80% of the iodine affinity (accuracy = ±2.5%) of each parent starch. The remaining iodine binding was explained by iodine affinity of the amylopectin. The total iodine affinity of the isolated linear and branched fractions commonly equalled slightly more than that of the starch; thus it would appear that complete liberation and dispersion of the fractions makes the chains more accessible to reaction with iodine.

Table 4. Properties of Amylopectins of High-Amylose Corn Starch

Breeding	Starch % „Apparent Amylose"[1]	Steep	Yield % Starch	[η] 1 N KOH	Iodine Affinity Mg I_2/g	% Amylose Equivalent[1]	β-Amylose Conversion
Hybrid	27	H_2O	72[2]	1.80	10	5	56%
Hybrid	50	H_2O	58	1.24	53	26	
			58[3]	1.21	56		
Hybrid	57	SO_2	40	1.30	—	29	
Inbred	61—65	H_2O	45	1.22	82	41[4]	
			44[5]	1.23	80		
Inbred	66	H_2O	43	0.92[6]	82	40	
Inbred	68	SO_2	37	1.23	—	28	
Inbred	68	H_2O	42	1.18	82.3	41	73
Inbred	71	H_2O	39	1.22	88	—	75

[1]) Spectrophotometric analysis of iodine binding (10).

[2]) Separated according to MONTGOMERY and SENTI (11).

[3]) Cf. Exp. 3, Table 3.

[4]) A part of sample was isolated after 8 hours reflux with Pentasol in aqueous solution buffered to p_H 6.6 and showed no change in iodine binding (± 2%).

[5]) Cf. Exp. 6, Table 3.

[6]) [η] prior to dialysis, 1.22; dialyzed sample dispersed under nitrogen in dimethyl sulfoxide, precipitated from water with ethanol and re-isolated, 1.20.

By means of the fractionation procedure adopted it was possible to explore the entire series of high-amylose corn starches on hand regardless of the manner of steeping, level of amylose in the granule, or the physical properties of each starch. However, high-amylose corn starches having other genetic backgrounds may differ in properties and for this reason claims for the use of the fractionation procedure can only be made for the types of starch investigated.

Properties of Amylose

Amylose from high-amylose corn was defined as that portion of the starch which was precipitated by n-butanol as a crystalline complex. Amylose from the high-amylose starches used in this study resembled the dent corn component. Amylose 1, or 90 to 93 % of the total fraction, absorbed 200 mg I_2/g and had an intrinsic viscosity of 1.32 to 1.37 in 1 N potassium hydroxide (Table 1). As recorded in column 8, Table 1, the molecular weight of examples of amylose 1 from dent and high-amylose corn starches was about 300,000 when determined by light scattering. Comparison of amylose from one starch (61—65 % amylose) prepared with and without aqueous sulfur dioxide steeping indicated that sulfur dioxide steeping had little effect on amylose as shown by intrinsic viscosity values (Table 1) for amylose from water- and SO_2-steeped 61 to 65 % amylose starches.

The amylose 2 fraction of high-amylose corn starch formed a crystalline complex with n-butanol, which was more soluble in water than was the major fraction, amylose 1. Amylose 2 fractions (Table 1) absorbed from 165 to 175 mg I_2/g and were obtained in yields of 7 to 9 % of the total amylose isolated. This fraction of amylose, although low in iodine affinity, had fairly high intrinsic viscosity, as shown in the subfraction of one amylose 2 fraction in Table 2. It is possible that this amylose is partially branched; if so, the molecular weight of amylose 2 may be greater than that of amylose 1. Recent investigations carried out on the corresponding dent corn amylose subfraction indicated that this portion of the butanol-precipitable fraction has a higher molecular weight than does the relatively unbranched amylose[2]).

Properties of Amylopectin

Amylopectin was the portion of the high-amylose corn starch that failed to give an insoluble complex with n-butanol or Pentasol. Major differences in structure of the amylopectin component were observed to accompany increase in the amylose content of corn starch granules (Table 4). The amylopectin component of the high-amylose content starch differed markedly from the dent corn fraction in iodine affinity; this value of the component isolated from 71 % amylose starch was about 88 mg I_2/g as compared with 10 mg I_2/g for that from 27 % amylose or dent corn starch. It appears that the high iodine affinity is inherent in the amylopectin rather than resulting from associated amylose since refluxing of an aqueous dispersion of one of the amylopectins with Pentasol for 8 hours and allowing the mixture to cool produced no insoluble Pentasol-starch complex. The amylopectin, when isolated and examined, retained an iodine affinity of 82 mg I_2/g (Table 4).

We have been unable to confirm GREENWOOD's (6) finding that the high iodine binding of the amylopectin from 50 % amylose starch results from an associated amylose which can be separated by differential sedimentation after dispersion in alkali. Exp. 3, Table 3 was repeated with two additional extractions of the amylopectin gel in 0.5 N sodium hydroxide, each followed by neutralization and recovery of the amylopectin by centrifugation at 40,000 X g for 3 hours. The separated amylopectin was again dissolved in 0.5 N sodium hydroxide, neutralized and examined in the analytical centrifuge. More than 95 % of the amylopectin sedimented in 32 minutes at 40,000 X g. The iodine binding of this amylopectin, recovered in a parallel run in the preparative ultracentrifuge, was 51.4 mg I_2/g. This is only slightly less than the values reported in Table 4 for the 50 % amylose starch, indicating little, if any, separation of amylose of low molecular weight by repeated extraction with alkali. The iodine binding of the amylopectin appears, therefore, to be characteristic of its molecular structure.

The high iodine affinity of the amylopectin is indicative of increased branch length, at least in the external linear portion of the chains. Further evidence of such a structural change was obtained by the action of β-amylase on the amylopectin of 71 % amylose starch. The extent of conversion was 75 % as compared to 56 % for dent corn amylopectin. The β-amylase limit dextrin was isolated in theoretical yield and showed no inflection by potentiometric iodine titration.

Further evidence of increased branch length in exterior portions of the chains of high-amylose corn amylopectin, when compared to the dent corn component, is found in retrogradative characteristics. The former retrograded readily from aqueous solution at 25° C, thus resembling amylose and differing from the dent corn amylopectin which does not retrograde at this temperature. Retrogradation of an 0.5 % solution was reversible; the solid redissolves on heating to 98° C; in contrast, retrograded amylose does not redissolve on heating. Retrogradation of amylopectin at higher concentrations was not completely reversible. High-amylose corn amylopectin, whether retrograded or not, is readily soluble in alkali.

Intrinsic viscosities of the amylopectin fractions ranged from 0.92 to 1.30. These values are lower than those reported by MONTGOMERY and SENTI (11) for dent corn amylopectin and indicate that the molecular weight of this fraction of the high-amylose starches may be less than that of dent corn amylopectin.

The change in amylopectin properties and in apparent structure appears in this series of starches to be related to the amylose content of the starch. The possibility remains that amylopectin similar to dent corn starch may be encountered in high-amylose starches having different genetic backgrounds. Conversely, still

[2]) F. R. SENTI and G. E. BABCOCK: Presented at 138th meeting of Am. Chem. Soc., New York, N. Y., Sept., 1960, page 16 D, abstracts.

greater differences in structure also may be found in other starches even within the same range of amylose contents as studied here.

Relation of Data to Starch Properties

Differences in the amylopectin may help to account for the properties of high-amylose corn starch. For instance, the marked increase in gelatinization temperature may be caused by the structurally modified amylopectin as well as the higher level of amylose. The longer branches in the amylopectin of high-amylose starch would enable firmer association of the amylopectin molecules with each other and/or with amylose, thereby increasing resistance of the granule to swelling and gelatinization.

At present the information on structure and properties of the amylose and amylopectin components of high-amylose corn starch is incomplete. Nevertheless, two significant points already stand out. First, the similarity of amylose to dent corn amylose promises that the foundation of research on the latter can be extended without major change to high-amylose corn amylose. Differences observed in the new amylopectins present new areas for research. The full impact of the amylopectin structure on the properties of high-amylose corn starch has yet to be determined.

Acknowledgment

Appreciation is expressed to R. S. Knobloch for assistance in the processing and analytical work; to Mrs. Carol B. Burnett for the crystalline β amylase; to the Engineering and Development Laboratory for the high-amylose corn starch; to R. P. Bear and M. S. Zuber for samples of high-amylose corn; to R. Tobin for the light-scattering measurements of molecular weight of amylose fractions; to E. H. Melvin and C. A. Glass for the spectrophotometric measurements of starch-iodine reactivity; and to Mrs. Clara McGrew and Mrs. Bonita Heaton for microanalyses. Support for this research was provided by the Corn Industries Research Foundation, Inc., 1001 Connecticut Avenue, N. W., Washington, D. C.

Summary

To compare high-amylose corn starch and its fractions with ordinary or dent corn starch and its fractions, high-amylose corn starches selected at six different levels of amylose content (apparent amylose as shown by iodine affinity was 50 to 71 %) were separated into amylose and amylopectin. Separation was attained by the granule pretreatment-extraction procedure, used by Montgomery and Senti (11) for fractionation of dent corn starch, with some modification.

High-amylose corn amylose, isolated in yields accounting for 80 % of the iodine affinity of the starch, resembled dent corn amylose in molecular size, as shown by intrinsic viscosity and molecular weight by light scattering, and in iodine affinity. As the amylose content of the granule increased, amylopectin showed increased iodine affinity and the length of the outer branches of the molecule increased as evidenced by β-amylase conversion limits of 73 and 75 % for the amylopectins from the 68 and 71 % amylose starches, respectively.

Zusammenfassung

Um amylosereiche Maisstärke und ihre Fraktionen mit normaler oder aus Zahnmais hergestellter Stärke und deren Fraktionen vergleichen zu können, wurden amylosereiche Maisstärken mit sechs verschieden hohen Amylosegehalten (die auf Grund des Jodbindungsvermögens ermittelten Amylosegehalte lagen zwischen 50 und 71 %) in Amylose und Amylopektin aufgetrennt. Die Trennung wurde mit Hilfe eines modifizierten, vorbehandelnden Extraktionsverfahrens durchgeführt, welches Montgomery und Senti (11) zur Fraktionierung von Stärke aus Zahnmais angewendet haben.

Die Amylose, die aus amylosereicher Stärke in den Ausbeuten gewonnen wurde, die entsprechend einer 80 %igen Jodaffinität der Stärke berechnet worden waren, war der aus Zahnmais hergestellten Amylose hinsichtlich der Molekülgröße ähnlich wie durch Messung der Strukturviskosität und des im Lichtstreuungsverfahren bestimmbaren Molekulargewichtes gezeigt werden konnte. Dasselbe gilt für die Jodaffinität. Mit zunehmendem Amylosegehalt der Stärkekörner zeigte das Amylopektin ein erhöhtes Jodbindungsvermögen. Außerdem vergrößerte sich die Länge der äußeren Verzweigungen des aus Stärken mit 68 bzw. 71 % Amylosegehalt gewonnenen Amylopektins, wie die mit β-Amylase erhaltenen Abbaugrenzen von 73 und 75 % zeigten.

Résumé

Des amidons de maïs riches en amylose ayant six fortes teneurs différentes furent décomposés en amyloses et amylopectines. On pouvait de cette façon comparer l'amidon de maïs riche en amylose et ses fractions avec l'amidon normal ainsi qu'avce l'amidon obtenu du maïs de zéa. Les teneurs apparentes en amyloses déterminées par l'iode étaient de 50 et de 71 %. La séparation fut effectuée à l'aide d'un système modifié d'extraction préparatoire, qui avait été appliqué au fractionnement de l'amidon obtenu du maïs de zéa par Montgomery et Senti (11).

L'amidon riche en amylose isolé avec un rendement de 80 % (ce dernier étant calculé par l'affinité d'iode de l'amidon) était semblable à l'amylose obtenu du maïs de zéa au point de vue dimensions de la molécule. Ce résultat fut obtenu par mesure de la viscosité structurelle et par la détermination du poids moléculaire à l'aide de la lumière diffuse. Il en est de même pour l'affinité d'iode. Celle-ci va en croissant, pour l'amylopectine, avec la teneur en amylose des grains d'amidons. En outre on a pu observer une augmentation de la longueur des chaînes extérieures de l'amylopectine provenant des amidons ayant une forte teneur en amyloses de 68 resp. 71 % comme le montre les limites de convertibilité par la β-amylase situées à 73 % et 75 %.

Literature Cited

(1) ANDERSON, R. A., and V. F. PFEIFER: Cereal Chem. **36** (1959), 98—101.

(2) (a) BALLS, A. K., M. K. WALDEN and R. R. THOMPSON: J. Biol. Chem. **173** (1948), 9—19;
(b) OLSON, W. J., R. EVANS and A. D. DICKSON: Cereal Chem. **21** (1944), 533—539.

(3) BATES, E. L., D. FRENCH and R. E. RUNDLE: J. Amer. Chem. Soc. **65** (1943), 142—148 as modified by WILSON, E. J., T. J. SCHOCH and C. S. HUDSON: J. Amer. Chem. Soc. **65** (1943), 1380.

(4) DEATHERAGE, W. L., M. M. MACMASTERS and C. E. RIST: Trans. Amer. Assoc. Cereal Chem. **13** (1955), 31.

(5) DEATHERAGE, W. L., M. M. MACMASTERS, M. L. VINEYARD and R. P. BEAR: Cereal Chem. **31** (1954), 50—52.

(6) GREENWOOD, C. T., and J. THOMSON: Chem. and Ind. (London) No. 35 (1960), 1110—1111.

(7) KRAMER, H. H., R. L. WHISTLER and E. G. ANDERSON: Agron. J. **48** (1956), 170;
KRAMER, H. H., P. L. PFAHLER and R. L. WHISTLER: *ibid.*, **50** (1958), 207—210.

(8) LANSKY, S., M. KOOI and T. J. SCHOCH: J. Amer. Chem. Soc. **71** (1949), 4066—4075.

(9) MEITES, L., and T. MEITES: Anal. Chem. **20** (1948), 984 bis 985.

(10) MELVIN, E. H., and C. A. GLASS: In preparation for publication; a modification of method of MCCREADY, R. M., and W. Z. HASSID: J. Amer. Chem. Soc. **65** (1943), 1154.

(11) MONTGOMERY, EDNA M., and F. R. SENTI: J. Polymer Sci. **28** (1958), 1—9.

(12) SCOTT, TROY A., Jr., and EUGENE H. MELVIN: Anal. Chem. **25** (1953), 1656—1661.

(13) SOMOGYI, M.: J. Biol. Chem. **160** (1945), 62.

(14) (a) VINEYARD, M. L., and R. P. BEAR: Maize Genetics Cooperation News Letter **26** (1952), 5;
VINEYARD, M. L., R. P. BEAR, M. M. MACMASTERS and W. L. DEATHERAGE: Agron. J. **50** (1958), 595—598;
(b) BEAR, R. P., M. L. VINEYARD, M. M. MACMASTERS and W. L. DEATHERAGE: *ibid.*, 598—602.

(15) WOLFF, I. A., B. T. HOFREITER, P. R. WATSON, W. L. DEATHERAGE and M. M. MACMASTERS: J. Amer. Chem. Soc. **77** (1955), 1654—1659.

(16) ZUBER, M. S., C. O. GROGAN, W. L. DEATHERAGE, J. E. HUBBARD, W. E. SCHULZE and M. M. MACMASTERS: Agron. J. **50** (1958), 9;
ZUBER, M. S., C. O. GROGAN, V. L. FERGASON, W. L. DEATHERAGE and M. M. MAC MASTERS: Cereal Chem. **37** (1960), 212—217.

Address of the Authors: F. R. Senti, Edna M. Montgommery and K. R. Sexson, Northern Regional Research Laboratory, Peoria, Illinois (USA).

This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(Arrived: 5. 4. 1961).

Neuere Beiträge zur Chemie der Stärkefraktionen. X.[1]) Über die Konformation der Glucopyranosidringe der Amylose[2])

Von J. HOLLÓ, J. SZEJTLI und M. TÓTH, Budapest (Ungarn)

Herrn Prof. Dr. M. Samec, Ljubljana, aus Anlaß seines 80. Geburtstages gewidmet.

Allgemeines über die Konformation der Glucopyranosidringe

Der Ausdruck „Konformation" wurde von HAWORTH (1) im Zusammenhang mit Zuckern bereits im Jahre 1929 verwendet; mit der Aufklärung der tatsächlichen Konformation verschiedener Zuckerarten begann man sich aber erst in den letzten Jahren zu beschäftigen(31). Die auf diesem Gebiet erzielten Resultate sind noch recht lückenhaft und in vielen Fällen widersprechend. Zweck der vorliegenden Arbeit ist es, die bisherigen Ergebnisse zusammenzufassen, auf Grund unserer eigenen Experimente weitere Daten zu liefern und das Problem der Konformation der Glucopyranosidringe der Amylose in den Vordergrund zu rücken, da man von ihm mit Fug und Recht erwarten kann, daß es zur Deutung von bisher noch unerklärlichen Tatsachen verhilft.

In den früheren Formeln für die Monosaccharide wurde ihre Raumstruktur überhaupt nicht berücksichtigt. Die HAWORTH'sche Formel stellt den Ring bereits in drei Dimensionen dar, aber auch dies drückt die tatsächlichen Verhältnisse noch nicht gehörig aus. Durch stereochemische Untersuchungen wurde es klar, daß die Ringverbindungen im allgemeinen nicht koplanär sind, d. h., daß die ringbildenden Atome nicht auf einer Ebene liegen. So hat z. B. der Cyclohexanring entweder die Form eines Sessels (chair) oder einer Wanne (boat) (Abb. 1). REEVES (2) zufolge sind für

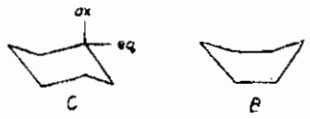

Abb. 1. Die Konformationen des Cyclohexanringes.

spannungsfreie Pyranosidringe zwei Sesselformen und sechs Wannenformen denkbar. Dies sind die acht Grundformationen (Abb. 2). Von den einzelnen Konformationen verhalten sich je zwei wie Bild und Spiegelbild zueinander.

[1]) Der IX. Teil ist erschienen in: Die Stärke **13** (1961), 32.
[2]) Die 33. Mitteilung des Instituts für Landwirtschaftlich-Chemische Technologie, Budapest, über die Erforschung von Polysacchariden.